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**Effects of low-dose doxycycline on cytokine secretion in human monocytes stimulated with *Aggregatibacter actinomycetemcomitans***

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## Abstract

Doxycycline is an antibiotic used in the treatment of a variety of inflammatory conditions, including periodontitis. Apart from its antimicrobial properties, this drug also has independent anti-inflammatory effects at sub-antimicrobial doses. The present study aimed to investigate the effects of low-doses of doxycycline (LDD) on cytokine production by human monocytic cells challenged with the periodontal pathogen *Aggregatibacter actinomycetemcomitans*, for up-to 6 hours. The simultaneous regulation of twelve cytokines were measured by a Human Cytokine Array Kit. To validate the array findings, selected cytokines were also measured by enzyme-linked immunosorbant assay (ELISA). *A. actinomycetemcomitans* stimulated the production of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 by the cells after 6 hours of challenge, and doxycycline significantly inhibited this effect. The kinetics of this regulation demonstrated an early (within 2 hours) and significant ( $P<0.05$ ) inhibition of pro-inflammatory cytokines, with a mild (0.5-fold) up-regulation of the anti-inflammatory cytokine IL-10. The results indicate that LDD acts as an anti-inflammatory agent in human monocytic cells stimulated with *A. actinomycetemcomitans*. This model provides clear evidence that some of the clinically proven benefits of LDD may be related to its ability to regulate inflammatory mediator release by monocytic cells. This property may contribute to the clinically proven benefits of this antibiotic as an adjunctive treatment for periodontitis.

**Keywords:** low-dose doxycycline, cytokines, periodontal disease, *Aggregatibacter actinomycetemcomitans*, inflammation, monocytes, MonoMac-6

## 1. Introduction

Periodontitis is an inflammatory condition caused by the accumulation of bacteria on tooth surfaces in the form of oral biofilms (1, 2). More than 700 bacterial species colonize the subgingival area but studies suggest that the presence of relatively few key species may be causally related to periodontitis. *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) is an important pathogen in severe and recurrent forms of this disease (3, 4). *A. actinomycetemcomitans* has been implicated as a key etiological agent in localized aggressive periodontitis, where this species can be detected in nearly 90 % of the patients, and in severe chronic periodontitis, where it may be present in between 30% - 50 % of patients (5-7). This bacterial species possesses many virulence factors that are thought to be involved in the processes of alveolar bone loss and connective tissue destruction (8-11). Although, *A. actinomycetemcomitans* is primarily considered as an oral pathogen, on occasions the organism can be detected in the systemic circulation (12, 13). The levels of *A. actinomycetemcomitans* have been shown to correlate with an excessive host inflammatory response in susceptible individuals as characterized by enhanced concentrations of pro-inflammatory or reduced levels of anti-inflammatory cytokines such as interleukin (IL)-10 (14-18).

The production of cytokines in response to periodontal bacteria includes contributions from a variety of cell types, but it is clear that monocytes are one of the most important sources within the gingival tissues (3, 19, 20). Monocytes secrete a wide variety of cytokines including IL-1  $\beta$ , tumor necrosis factor (TNF)- $\alpha$  and IL-18 in response to periodontal pathogens which in turn play a major role in the initiation of protective immune responses in the periodontium (21-24). However, extensive release of pro-inflammatory cytokines such as TNF- $\alpha$  may harm the host by accelerating inflammation and activating bone and tissue destruction (25, 26). It is very likely that imbalances in cytokine production determine the extent of periodontal tissue destruction in susceptible individuals. Therefore modulating immunoinflammatory response to the bacterial exposure is an important concept in the treatment of various inflammatory diseases including periodontal disease (27, 28).

Doxycycline, a member of the tetracycline family of antibiotics, has been approved by the United States Food and Drug Administration (FDA) as a host modulatory agent for use as an adjunct to conventional therapy for the treatment of chronic periodontitis

(7, 29, 30). Approval of doxycycline, more specifically subantimicrobial dose doxycycline was based on its ability to inhibit the activity of matrix metalloproteinases (MMPs). The MMPs are thought to be responsible for degradation of periodontal connective tissue (31). It is also known that MMPs are induced in response to a range of cytokines, including IL-1 $\alpha$  and TNF- $\alpha$  (32, 33).

However, it is unclear to what extent this low-doses of doxycycline is acting directly on MMP activity or if this reduced activity reflects a generalised anti-inflammatory effect (34-37). Improving our knowledge of host cytokine regulation in response to bacterial challenge is fundamental to understanding the clinical course of the disease and developing novel treatment strategies for periodontitis (38, 39). The clinical impact of LDD in modulating MMP activity is well-described, but its impact on the production of cytokines by monocytes is incomplete. Hence, this study aimed to investigate the effect of LDD on the regulation of a broad spectrum of inflammatory mediators by monocytic cells challenged with *A. actinomycetemcomitans*, as an *in vitro* model for periodontal infection.

## **2. Materials and Methods**

### **2.1 Bacterial stimuli and materials**

Supernatants were obtained from cultures of *A. actinomycetemcomitans* Y4. Cultures were grown in Tryptic Soy Broth (Difco) with 0.6% yeast extract in an anaerobic chamber containing an atmosphere of 80% v/v N<sub>2</sub>, 10% v/v H<sub>2</sub>, and 10% v/v CO<sub>2</sub>. Cultures were grown to approximately 5x10<sup>7</sup> colony forming units/ml and harvested by centrifugation at 10,000g for 15 min at 4°C. The culture supernatants were collected, filter-sterilized (0.2 $\mu$ m) and stored at -80°C until used. The supernatant was directly diluted in culture medium to a final dilution of 1:250, a concentration which has been shown to induce cytokine production in the absence of cell toxicity (22). Commercially obtained lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 (Sigma-Aldrich, UK) was used as a positive control. Doxycycline hyclate (Sigma-Aldrich, UK) was dissolved in distilled water (stock of 50 mg/ml) and filter-sterilized (0.2 $\mu$ m).

### **2.2 Cell lines, cell culture, and reagents**

The myelomonocytic cell line, MonoMac-6 (40) was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). MonoMac-6 cells were cultured in RPMI-Glutamax (Gibco BRL Life Technologies, UK) supplemented with 10% fetal bovine serum (Bio-Whittaker, Maryland), 1% nonessential amino acids (Gibco BRL), 1% sodium pyruvate (Gibco BRL) and 9 µg/ml bovine insulin (Sigma-Aldrich, UK). The assays were performed with cells at a density of  $1 \times 10^6$  cells per ml.

### **2.3 Isolation of monocytes from PBMCs**

Peripheral blood was obtained from healthy adult volunteers by venous puncture following approval of the protocol by the East London and City Health Authority London Research Ethics Committee. Human peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences) according to the manufacturer's instructions. Purified monocytes were isolated from the PBMCs as described previously (22). Briefly, the cell layer containing mononuclear cells was collected and washed twice with PBS containing EDTA (2mM) and bovine serum albumin (2%). Purified monocytes were isolated using negative selection that involves depletion of T cells and B cells using the Monocyte Isolation Kit II (Miltenyibiotec, Bergisch Gladbach, Germany). The purity of isolated monocytes was evaluated by flow cytometry on a FACScan flow cytometer (Becton Dickinson) using a CD14 FITC-conjugated antibody. Purified monocytes were re-suspended in RPMI-Glutamax (Gibco BRL Life Technologies, UK) supplemented with 10% fetal bovine serum (Bio-Whittaker, Maryland). The assays were performed with cells at a density of  $1 \times 10^6$  cells per ml.

### **2.4 Cell viability assays**

Cell viability was determined by measurement of lactate dehydrogenase (LDH) release using the CytoTox 96 non-radioactive cytotoxicity assay (Promega, UK). In brief, MonoMac-6 cells or primary human monocytes were exposed to LDD (5-25 µg/ml) or bacterial supernatants then incubated for 6 hours at 37°C. 50 µl/well supernatant was carefully removed and transferred into an optically clear 96-well plate. Reaction solution was added followed by incubation for 30 min in darkness. The enzyme reaction was then stopped by the addition of 1N HCl and the absorbance was measured using a spectrophotometric plate reader at 490nm (Model 680, Bio-Rad

Laboratories). The activity of the enzyme released from damaged cells into the supernatant was measured, and was expressed as a percentage of the total LDH activity released from cells lysed by exposure to 0.1% Triton X-100 for 45 min.

## **2.5 Measurement of cytokine production**

The cells were seeded in 96-well plates at density of  $1 \times 10^6$  cells/ml and incubated in the presence of bacterial supernatant from *A. actinomycetemcomitans* strain Y4 with or without doxycycline at indicated concentrations (5-25  $\mu\text{g/ml}$ ) up to 6 hours at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  atmosphere. To assay cytokine production, the cell free culture supernatants were collected by centrifuging at 1000 rpm for 5 minutes and stored at  $-80^\circ\text{C}$  until the cytokine assays were performed. The concentrations of  $\text{TNF-}\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$  and IL-6, IL-8 were determined by an enzyme-linked immunosorbant assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader (Model 680, Bio-Rad Laboratories) with a wavelength correction set at 570nm. A standard curve was generated using a four parameter logistic (4-PL) curve-fit (Microsoft Office Excel) for each set of samples assayed. The values of the samples were assigned according to the standard curve. The simultaneous regulation of 12 cytokines was assessed in the culture supernatants using multiplex immunoassays. The Proteoplex 16 well Human Cytokine Array Kit, (Merck Biosciences, UK) was used for the assessment of  $\text{TNF-}\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12, IFN- $\gamma$  and GM-CSF. The minimum detectable concentration in this assay was 5 pg/ml for each cytokine. The use of both ELISA and Proteoplex methods in the investigation of these inflammatory mediators provides a better validation and ensures the methodological repeatability of the results.

## **2.6 Statistical analysis**

The significance of differences between the control (*A. actinomycetemcomitans*) and test groups (*A. actinomycetemcomitans*  $\pm$  LDD) were assessed by one-way ANOVA and Bonferoni Post tests, using the GraphPad Prism Version 4.00 software.  $P < 0.05$  values were considered statistically significant. The data are expressed as mean  $\pm$  standard deviation (SD) in each group.

### 3. Results

Firstly, the potential cytotoxic effects of LDD (up to 25 µg/ml) were investigated. There was no significant increase in LDH release, as a marker of cell damage, in cells co-cultured with LDD, compared to the control (Table 1). Hence, these concentrations were deemed suitable for further experiments. Exposure of MonoMac-6 cells to culture supernatants of *A. actinomycetemcomitans* for 6 h significantly induced the production of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  by the cells (Table 2). To determine the effects of LDD on the production of these pro-inflammatory cytokines, two different doses of the drug (10 µg/ml and 25 µg/ml) were added to the cells at the same time as the challenge with the culture supernatants. A dose-dependent inhibition in the production of these cytokines was observed (Table 2), the four studied interleukins were the most significantly inhibited. To confirm the effects of LDD (5 µg/ml - 25 µg/ml) on the production of proinflammatory cytokines by primary monocytes, either culture supernatant from *A. actinomycetemcomitans* or *E. coli* LPS (10 ng/ml) were concomitantly added to the cells, for 6 h. Both stimulants induced a marked increase in the production of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 by the cells, whereas LDD caused a dose-dependent inhibition of this effect (Figures 1 A-D). Maximal inhibition was observed with 25 µg/ml doxycycline by 70%, 74%, 84% and 85%, respectively. To investigate the kinetics of cytokine inhibition by doxycycline, MonoMac-6 cells were challenged with *A. actinomycetemcomitans* supernatants for up-to 4 h in the presence of LDD (10 µg/ml). After this 4 h period, a cytokine array was used for the analyses (Figure 2). The levels of IL-2, IL-4, IL-7 and IFN- $\gamma$  secretion were low or undetectable (< 5 pg/ml). A reduction in IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 levels was demonstrated already by 2 h, whereas a change in TNF- $\alpha$  levels was not detected until 4 h. TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 production was inhibited by 27%, 67%, 43%, 97%, and 98%, respectively (Figure 2). In contrast, the production of IL-10 was increased by 0.5-fold whereas that of IL-12 by 8-fold, in the presence of LDD, over 4 h (Figures 2F and 2G). 1 h following challenge with *A. actinomycetemcomitans*, the GM-CSF levels were increased by 2-fold and maintained unchanged over 4 h (Figure 2H). Interestingly, the presence of LDD further enhanced by 1.7-fold the *A. actinomycetemcomitans*-induced GM-CSF levels observed after 1 h, but this difference was ablated after a further 4 h (Figure 2H).



#### 4. Discussion

The present study demonstrates that LDD doxycycline inhibits the production of several pro-inflammatory cytokines in human monocytic cells stimulated with *A. actinomycetemcomitans* culture supernatants. The importance of these findings is that, apart from its inhibitory effect on MMP activity, LDD also appears to exert more general anti-inflammatory effects, manifested as reduction of pro-inflammatory cytokine production by monocytes in the presence of *A. actinomycetemcomitans*. Thus LDD may have marked modulatory effects on the inflammatory response through a wide range of actions. For example, inhibition of IL-1 $\beta$ , TNF- $\alpha$  and IL-6 may result in decreased bone resorptive activity, whereas inhibition of IL-8 may attenuate the influx of inflammatory cells into the affected area (41). These findings are in agreement with previous reports showing that LDD inhibits the release of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 by human macrophages induced by isolated *A. actinomycetemcomitans* LPS (42). In other experimental systems involving *Chlamydia trachomatis* infection of monocytic THP-1 cells, it has been shown that the production of these four pro-inflammatory cytokines is reduced in response to LDD (43). Taken together, it can be deduced that LDD has general anti-inflammatory properties. Inhibition of bacteria-induced cytokine secretion by doxycycline occurred in a dose-dependent manner, within a concentration range between 5-25  $\mu\text{g/ml}$ , which did not show any evidence of cytotoxicity. LDD concentrations of 2 -10  $\mu\text{g/ml}$  have been reported in GCF, gingival tissue or plasma after oral administration of this drug (44). Thus, the concentration of doxycycline used in this study is approximately within the concentration range of the drug found in tissues and biological fluids of patients after oral administration. Hence the present results are potentially of direct biological significance *in vivo*. The exact mechanism of LDD-induced inhibition of cytokines by monocytes is not clear, although related studies have indicated that its effects occur via a post-transcriptional or translational mechanisms (45, 46). The kinetics experiments showed that LDD displayed an early inhibitory action on IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 production within 2 hours, whereas a change in TNF- $\alpha$  level was only observed at 4 h. These early responses are likely to indicate a direct effect of the drug on pro-inflammatory cytokine production, rather than mediation by other secreted factors. However, IL-12 production displayed a different response profile, as this was rapidly up-regulated in the presence of LDD. It has been proposed that an

increase in IL-12 production could be an event associated with the inhibition of pro-inflammatory cytokines, such as TNF- $\alpha$  (47). IL-12 is considered a link between innate and adaptive immunity (48, 49), and may be related to the pathogenesis of periodontal disease (50-52). Another interesting observation in the present study is that the production of the anti-inflammatory cytokine IL-10 was increased in response to LDD, possibly potentiating its beneficial effects to the host (53). This is an important finding as the relative IL-1 $\beta$ /IL-10 ratio has been shown to be higher in aggressive periodontitis patients than periodontally healthy subjects (54). Modification of this ratio by LDD may help to improve the imbalance between pro- and anti-inflammatory cytokines in periodontitis. The levels of IL-2, IL-4, IL-7 and IFN- $\gamma$  secretion were low or undetectable (<5 pg/ml). This is perhaps not surprising since earlier studies have shown that monocytes isolated from periodontitis patients do not produce IL-2 and IL-4 (55). Early induction of GM-CSF was observed in response to *A. actinomycetemcomitans* and further enhanced transiently by LDD. In support of these findings, *A. actinomycetemcomitans* cytolethal distending toxin was not able to induce production of GM-CSF by human PBMCs (56). Increased serum levels of IFN- $\gamma$  have been associated with *A. actinomycetemcomitans* infection but natural killer cells of the innate immune system are thought to be their primary source (57). In conclusion, the present results indicate that LDD attenuates the production of a wide range of pro-inflammatory cytokines by human monocytes, induced by the major periodontal pathogen *A. actinomycetemcomitans*. Hence, within the limitation of this *in vitro* work, the present findings may lend support that the clinically proven benefits of this drug may be related to its ability to regulate inflammatory mediator release by monocytic cells, and not only due to the attenuation of MMP activity. This may well be in line with recent findings demonstrating that administration of LDD as an adjunct to non-surgical periodontal treatment stabilizes the inflammatory response by suppressing the levels of pro-inflammatory cytokines and enhancing anti-inflammatory cytokines in GCF (36).

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## Tables

**Table 1**

### LDH release

#### % of total LDH

	Control	+Dox 10 µg/ml	+Dox 25 µg/ml
<b>MonoMac-6</b>	14.7 ± 2.3	10.3 ± 3.9	12.7 ± 0.9
<b>Primary monocytes</b>	13.6±1.2	13.4 ±0.7	14.6±1.6

Effects of doxycycline on cell viability. MonoMac-6 cells or primary human monocytes were incubated with the indicated two doses of doxycycline for 6 hours. Cytotoxicity was determined by the release of the cytosol enzyme lactate dehydrogenase (LDH), expressed as a percentage of the total (intracellular + extracellular) LDH activity. The data is expressed as mean ± standard deviation (n = 3).

**Table 2**

### Cytokine

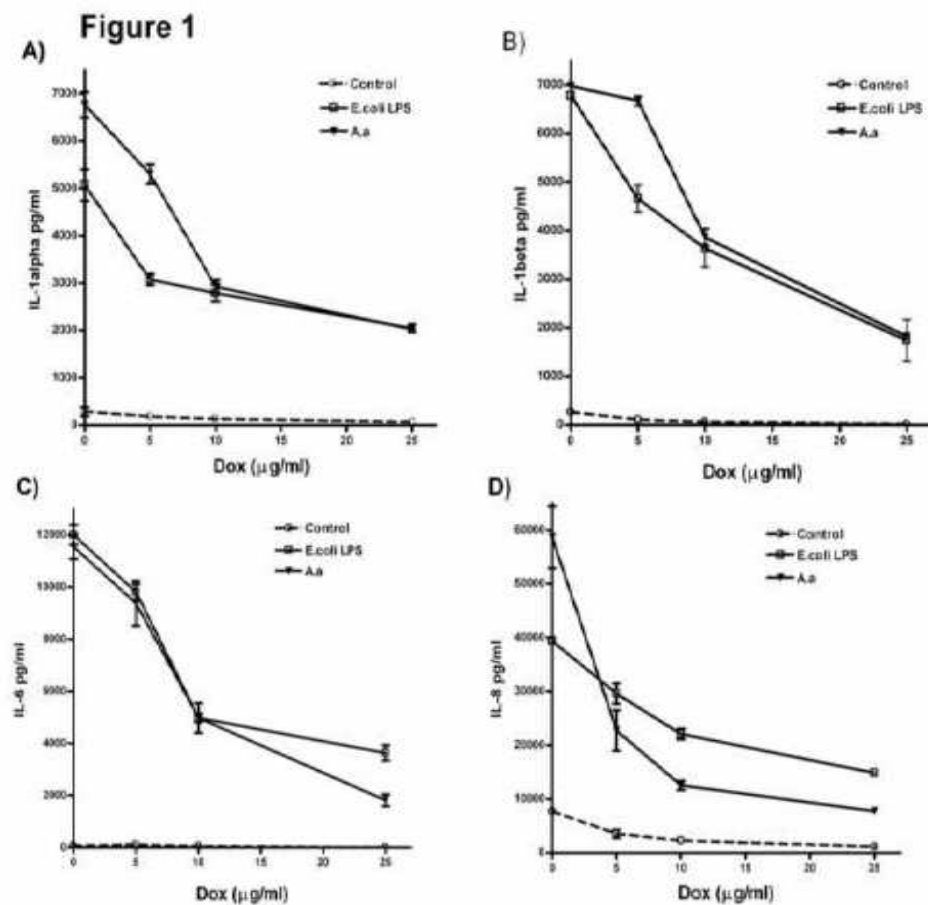
	Control	A.a	A.a+Dox 10µg/ml	A.a+Dox 25µg/ml
<b>IL-1α</b>	8.7 ± 0.7 *	496.6 ± 24.1	21.4 ± 6.0 *	10.2 ± 2.3 *
<b>IL-1β</b>	17.5 ± 1.1 *	1120.3 ±129.7	228.0 ± 20.3*	149.8 ± 13.3 *
<b>IL-6</b>	11.2 ± 4.5 *	271.1 ± 6.4	6.4 ± 3.9 *	6.1 ± 2.5 *
<b>IL-8</b>	554.3± 99.7 *	3110.8 ± 153.1	37.6 ± 17.6 *	27.6 ± 23.0 *
<b>TNF-α</b>	78.24 ± 3.5*	3142.0 ± 51.6	1395.1 ± 47.9 *	837.3 ± 19.8 *

MonoMac-6 cells were exposed to culture supernatants from *A.actinomycetemcomitans* in the presence or absence of doxycycline (10 or 25 µg/ml). After 6 hours of challenge, cell-free culture supernatants were collected to assess cytokine release by ELISA. The data is expressed in pg/ml represent the means ± standard deviation (n=3) Asterisk (\*) represents statistically significant differences as compared to the *A. actinomycetemcomitans*-challenged group ( $P < 0.05$ ).



## Figure Legend

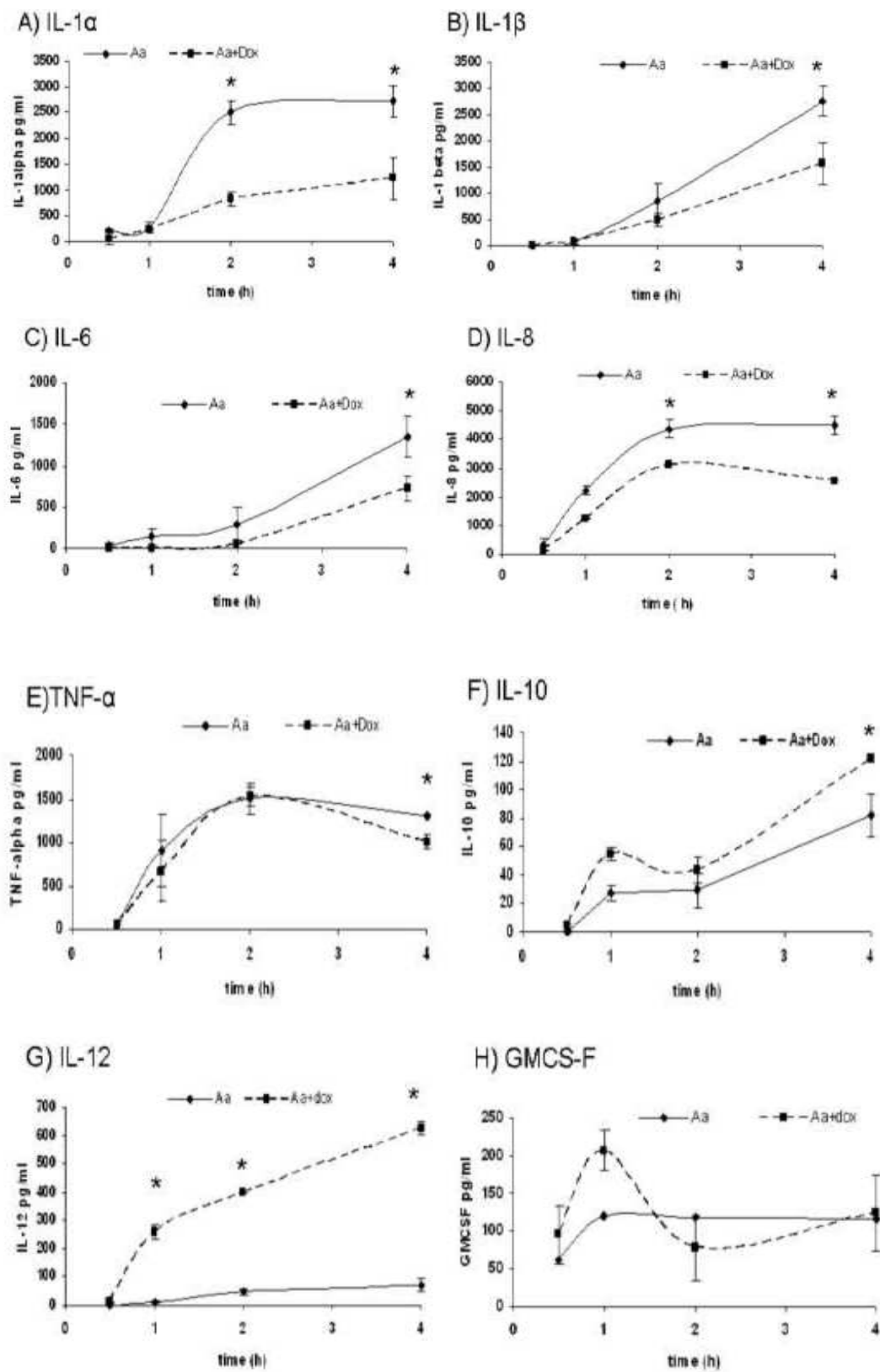
Figure 1



The levels of IL-1α, IL-1β, IL-6 and IL-8 in human monocytes regulated on activation by *A. actinomycetemcomitans* or *E.coli* LPS in the presence or absence of doxycycline (Figures A-D). Freshly isolated CD14<sup>+</sup> monocytes were plated at a density of 1 x 10<sup>6</sup> cells/ml in 96-well dishes. Cells were exposed to culture supernatants from *A.a* or *E.c* LPS (10 ng/ml) in the presence or absence of doxycycline (5 to 25 μg/ml). After 6 h incubation, cell-free supernatants were collected to assess cytokine release by ELISA. The data are means ± SD (n=3) representative of one of three separate experiments. Asterisk (\*) represents statistically significant differences as compared to the *A. actinomycetemcomitans* or *E.coli* LPS challenged group ( $P < 0.05$ ).

Figure 2

Figure 2



Kinetics of cytokine release in the presence of doxycycline. MonoMac-6 cells were exposed to culture supernatants of *A. actinomycetemcomitans* (A.a) in the presence or absence of doxycycline (10 µg/ml). Cell-free supernatants were collected at indicated time points (0.5 h, 1 h, 2 h, 4 h) to assess cytokine release by a cytokine array. Levels of detectable cytokines are shown for IL-1 $\alpha$  (A), IL-1 $\beta$  (B), IL-6 (C), IL-8 (D), TNF- $\alpha$  (E), IL-10 (F), IL-12 (G), and GM-CSF (H). Asterisk (\*) represents statistically significant differences of the two groups at any time-point ( $P < 0.05$ ).